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MINIREVIEW

A Tangled Web: Regulatory Connections between Quorum Sensing and Cyclic Di-GMP

Disha Srivastava and Christopher M. Waters

Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan, USA

Bacteria sense and respond to environmental cues to control important developmental processes. Two widely conserved and important strategies that bacteria employ to sense changes in population density and local environmental conditions are quorum sensing (QS) and cyclic di-GMP (c-di-GMP) signaling, respectively. The importance of these pathways in controlling a broad variety of functions, including virulence, biofilm formation, and motility, has been recognized in many species. Recent research has shown that these pathways are intricately intertwined. Here we review the regulatory connections between QS and c-di-GMP signaling. We propose that the integration of QS with c-di-GMP allows bacteria to assimilate information about the local bacterial population density with other physicochemical environmental signals within the broader c-di-GMP signaling network.

Decoding the language of chemical signaling in bacteria and the mechanisms by which these signals control coordinated behavior impacts our understanding of the role of bacteria in human health, the environment, and industrial processes. Bacteria exist in communities and often perform coordinated activities corresponding to functions such as production and secretion of extracellular enzymes, luminescence, biofilm formation, and virulence. One such mechanism that regulates communal behavior is the process of cell-cell signaling known as quorum sensing (QS). QS controls density-dependent gene expression via the secretion and detection of chemical signals known as autoinducers (AIs) to sense the local population density (48). At low cell density, the concentration of AIs is low. As the bacteria accumulate, the relative concentrations of these signals increase, leading to formation of a minimal bacterial population referred to as a quorum. Increases in concentrations of AIs switch gene expression from an individualistic low-cell-density lifestyle to a coordinated high-cell-density state (48). Each bacterium controls specific phenotypes associated with low and high cell densities, depending on the specific biology and environment of the organism. For example, in *Vibrio cholerae*, biofilm genes are expressed only at the low-cell-density state whereas *Pseudomonas aeruginosa* induces biofilm formation in the high-cell-density state (11, 13, 21). QS is typically considered to mediate intraspecies communication, although there is evidence that interspecies communication also occurs (59). In essence, QS can be seen as a mechanism that allows bacteria to sense and adapt to the distinct environments of low and high cell densities.

Another important signal in bacteria that allows adaption to different environments is the second messenger signal, cyclic di-GMP (c-di-GMP). C-di-GMP, predicted to be present in 85% of all bacteria, controls the switch between biofilm formation and motility (18). The role of c-di-GMP in controlling the transition from a motile to a sedentary state has been observed in many bacteria, including but not limited to *P. aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *V. cholerae* (27). However, it is clear that c-di-GMP impacts a wide array of other fundamental bacterial behaviors, including cell cycle propagation, development, fimbrial synthesis, type III secretion, RNA modulation, stress response, bacterial predation, and virulence (14, 26, 30, 31, 38, 60, 72). It is likely that this list will continue to grow.

C-di-GMP is synthesized from two GTP molecules by diguanylate cyclase (DGC) enzymes containing GGDEF domains consisting of approximately 170 amino acids (54). Conversely, c-di-GMP is degraded by phosphodiesterase (PDE) enzymes containing EAL or HD-GYP domains that are approximately 250 amino acids in length (55). Proteins containing GGDEF and EAL domains or HD-GYPs are typically modular in nature, with the enzymatic domain linked to various amino-terminal sensory domains. These sensory domains respond to environmental or host-derived cues to control the downstream enzymatic activity. Thus far, only a few specific environmental signals, including norspermidine, oxygen, light, nitric oxide, and arginine, have been identified (3, 7, 33, 34, 46, 67).

As opposed to QS, wherein one or a small number of cascades converge to control responses, c-di-GMP signaling employs multiple signaling pathways. A striking feature of c-di-GMP signaling is that many bacteria encode a wide array of c-di-GMP synthesis and degradation proteins. For example, *Escherichia coli* K-12 encodes 12 GGDEF-containing proteins, 10 EAL-containing proteins, and 7 proteins that have both a GGDEF domain and an EAL domain (18). Although the enzymatic domains are conserved, each of these proteins has a unique N-terminal sensory domain that is predicted to respond to a specific cue. The changes in levels of c-di-GMP are sensed by c-di-GMP receptor proteins or riboswitch RNAs which regulate downstream phenotypes (27). Interestingly, regulation of downstream phenotypes occurs at many levels, including transcriptional regulation, posttranscriptional modulation, and direct control of an enzymatic response or protein activity (37, 49, 50, 62).

Therefore, much like QS, c-di-GMP functions to integrate external inputs to allow bacteria to adapt and respond to changing

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Address correspondence to Christopher M. Waters, watersc3@msu.edu.

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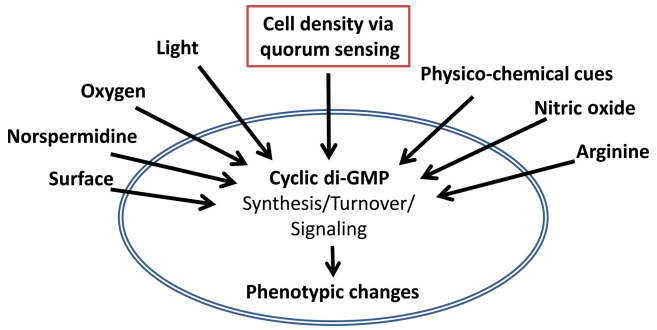

FIG 1 C-di-GMP signaling integrates information about local cell density through QS. The synthesis and degradation of c-di-GMP are controlled by multiple environmental signals to modulate downstream phenotypic changes. In this review, we argue that the information regarding local cell density transmitted by QS pathways is but one of many environmental signals that are ultimately integrated into the c-di-GMP signaling network composed of multiple signaling pathways (not depicted here) to allow bacteria to appropriately adapt and respond to different environments.

conditions. It is typically thought that these signals reflect the local physicochemical environment or other specific environmental cues (7, 27, 34, 67). In this review, we illustrate what is known about the connections between QS and c-di-GMP and argue that local cell density itself is one of the environmental cues sensed by the c-di-GMP network. We propose that the integration of these two signaling systems allows information about local cell density to be merged with other environmental cues into the broader c-di-GMP signaling pathway (Fig. 1).

CONTROL OF HD-GYP ACTIVITY BY QUORUM SENSING IN *XANTHOMONAS CAMPESTRIS*

In *Xanthomonas campestris*, a QS autoinducer (AI) signal directly regulates the activity of an HD-GYP enzyme, leading to a decrease in c-di-GMP levels. This system illustrates a direct interaction between a QS signal and a c-di-GMP signaling pathway. *X. campestris* synthesizes and responds to an AI termed Diffusible Signal Factor (DSF) that was recently determined to be *cis*-11-methyl-2-dodecenoic acid (70). This signal belongs to a family of AIs produced by *Xanthomonas* species and other related bacteria such as *Burkholderia cenocepacia* (26). The QS system of *X. campestris* stimulates the production of extracellular polysaccharide (EPS), which is itself a virulence factor in the plant host, and secretion of additional virulence factors at high cell density (26).

The genes involved in the *X. campestris* QS pathway are designated *rpfA* to *-G*, for their involvement in the regulation of pathogenicity factors (2). RpfB is a long-chain fatty acid ligase, which supplies DSF precursors to RpfF, an enoyl-coA hydratase, to synthesize DSF (2). DSF is directly sensed by the sensor kinase RpfC, which phosphorylates the HD-GYP protein RpfG only in the presence of DSF (Fig. 2). RpfC contains five transmembrane domains, a histidine kinase domain, a receiver (REC) domain, and a histidine phosphotransfer domain (57). The model for this system is that at low cell density in the absence of sufficient DSF, RpfC, the

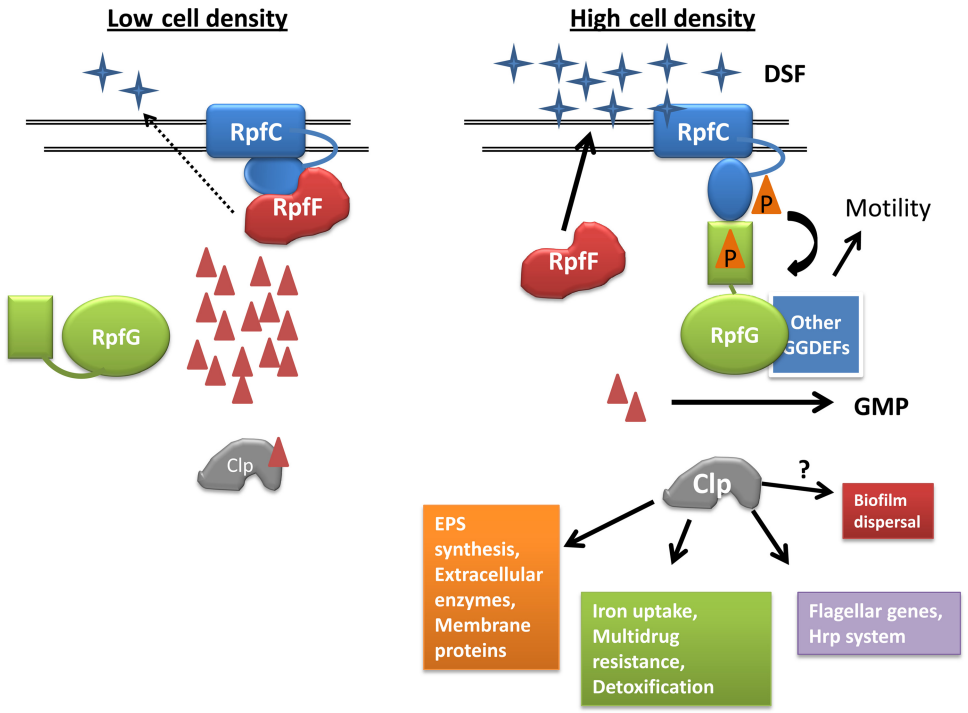

FIG 2 The QS system of *Xanthomonas campestris* modulates c-di-GMP levels in the cell. The AI signal, DSF, depicted as blue four-pointed stars, is synthesized by RpfF protein (red) and sensed by RpfC (blue), a membrane-bound histidine kinase protein. The double lines in Fig. 2 to 4 indicate the inner membrane. At low cell densities, when the concentration of DSF is low, RpfC interacts with RpfF and decreases its activity. In this state, the HD-GYP protein RpfG (green) is unphosphorylated and inactive. This leads to an increase in c-di-GMP levels (depicted by red triangles). C-di-GMP binds to the transcription factor Clp (gray) to abrogate its ability to bind DNA. At high cell density, RpfC binds to DSF, leading to phosphorylation of RpfG, which activates its PDE activity and decreases the c-di-GMP pool. RpfF no longer binds to RpfC and produces more DSF. The decrease in c-di-GMP activates Clp, which then induces target gene expression either directly or through modulation of other transcription factors. Clp can also promote biofilm dispersal through an unknown mechanism. RpfG also binds to and inhibits the activity of other GGDEF proteins.

sensor kinase, interacts with RpfF, the DSF synthase. This leads to a decrease in the production of DSF signal, since the activity of RpfF is reduced upon interaction with RpfC. At low cell density, the REC domain of RpfG is unphosphorylated, leading the HD-GYP domain to be enzymatically inactive (24). Thus, c-di-GMP levels are high at low cell density. However, at high cell densities, RpfC perceives DSF and undergoes autophosphorylation, which releases RpfF (24). RpfF then produces more DSF. Moreover, RpfC phosphorylates the receiver domain of RpfG, activating the C-terminal HD-GYP domain and reducing cellular c-di-GMP levels (52). Lowering of c-di-GMP levels stimulates virulence factor production through the transcription factor Clp (described below). Deletion of the genes that produce DSF, RpfC, or RpfG reduced the virulence of *X. campestris* in plant-based disease models (26). RpfG was the first HD-GYP protein shown to be a c-di-GMP PDE (52), and this system is the first in which QS and c-di-GMP were directly linked.

Interestingly, RpfG also interacts with several proteins containing GGDEF domains, and the consequences of this interaction are beginning to emerge (53). Yeast two-hybrid studies in *X. campestris* performed using RpfG as the bait identified eight GGDEF domain-containing proteins, two of which were shown to directly interact with RpfG *in vivo* using fluorescence resonance energy transfer analysis. Interaction of RpfG with these proteins required the HD-GYP domain and was influenced by DSF levels (53). This interaction was shown to be involved in the regulation of motility but not control of biofilms or extracellular enzyme secretion. It was suggested that control of biofilms and extracellular enzyme secretion occurs through a distinct pool of c-di-GMP also maintained by RpfG interaction with other GGDEF proteins (53). In a similar study in the related *Xanthomonas axonopodis*, 23 GGDEF domain-containing proteins were shown to interact with RpfG in a yeast two-hybrid assay, and some of these presumably interact with RpfG *in vivo* (1). These exciting results provide further evidence that localized c-di-GMP signaling is mediated by protein complex formation between c-di-GMP synthesis and degradation enzymes. Furthermore, sequestration of GGDEF domains by RpfG reveals another mechanism by which the DSF signaling pathway is further integrated into the broader c-di-GMP signaling pathway (1, 53).

The next issue was how the alterations in c-di-GMP levels control gene expression. Microarray studies revealed that DSF regulates a multitude of genes in *X. campestris* important in virulence corresponding to functions ranging from EPS production to flagellar biosynthesis, metal acquisition, and antibiotic resistance (25). One of the transcription factors regulated by DSF and induced upon c-di-GMP degradation was Clp (23, 62). Clp contains a cNMP binding domain and a DNA binding domain. Clp shares high homology with CAP, the classical cyclic AMP-dependent transcriptional activator that mediates catabolite repression (23). A *clp* deletion mutant showed altered regulation of EPS biosynthesis, protein metabolism, and extracellular enzymes, functions also regulated by DSF, RpfC, and RpfG, implicating Clp as the downstream regulator of the DSF QS pathway (Fig. 2) (23). Carbohydrate catabolism was not affected in the mutant, suggesting that Clp plays no role in that process (12).

Recent evidence showed that Clp responds to DSF signaling by directly sensing fluctuating c-di-GMP levels (62). Clp binding to c-di-GMP changes its conformation, abrogating its ability to bind DNA, which decreases the expression of Clp-regulated genes (9, 62). Therefore, at low cell density when c-di-GMP levels are high, Clp is inactive and its target genes are not expressed. Upon activation of RpfG at high cell density, the levels of c-di-GMP are reduced and Clp then induces expression of its target genes, leading to virulence factor expression (Fig. 2). The Clp homologues from *X. axonopodis* and *Stenotrophomonas maltophilia* were also shown to be c-di-GMP binding proteins, highlighting that this is a new class of c-di-GMP binding effectors that are found in other species (39, 62). It remains to be tested if the activities of these homologues are involved in QS pathways similar to those of *X. campestris*.

The beautiful research performed in *X. campestris* continues to offer fundamental insights into both QS and c-di-GMP signaling pathways. This system provided the first evidence that HD-GYP proteins degrade c-di-GMP, the first example of a c-di-GMP signaling protein directly sensing an AI molecule, and now the first description of apparent sequestration of c-di-GMP synthesis and degradation enzymes in protein complexes. *X. campestris* encodes 21 GGDEF, 4 EAL, 10 GGDEF-EAL, and 3 HD-GYP domain proteins (18). Therefore, RpfG is 1 of 38 predicted c-di-GMP synthesis/degradation enzymes in this bacterium. It is clear that the environmental "cue" recognized by the RpfC/RpfG signaling system, DSF, contains information about the local population density. As all of the 38 c-di-GMP synthesis/degradation enzymes likely respond to different cues, DSF is only one of many environmental signals that determine the overall levels of c-di-GMP within the cell. This regulatory architecture argues against the view that c-di-GMP is merely the regulatory mechanism by which QS exerts its effects but rather suggests that QS modulation of c-di-GMP allows bacteria to combine information in extracellular AIs with other pertinent environmental conditions to properly modulate the expression of downstream phenotypes (Fig. 1).

QS MEDIATED CONTROL OF C-DI-GMP REGULATES BIOFILM FORMATION IN *VIBRIO CHOLERAE*

The QS system of *V. cholerae*, the human pathogen that causes cholera, has been well characterized, although not every *V. cholerae* strain encodes a complete, functional QS system (32). This QS system possesses two parallel sensory circuits that respond to two specific AIs, a furanosyl borate diester named AI-2 and a hydroxylated alkyl ketone named CAI-1 (29) (Fig. 3). Signal perception of AI-2 in the periplasm by LuxPQ and CAI-1 by CqsS modulates a phosphorelay cascade that ultimately results in phosphorylation or dephosphorylation of LuxO, the central response regulator of the pathway (8, 48). At low cell density, the AI receptors function as kinases and LuxO is phosphorylated. Phospho-LuxO activates expression of four *qrr* small RNAs (sRNAs), which then repress expression of the master high-cell-density transcription factor HapR by destabilizing the *hapR* mRNA (40, 66). HapR is the master high-cell-density QS transcriptional regulator in *V. cholerae*, and it both activates and represses high-cell-density QS target genes (47). Recently, the transcription factor AphA was shown to be the master low-cell-density QS regulator of *V. cholerae* (51). At high cell density, interaction of the receptors with AIs switches their activity to phosphatases, leading to dephosphorylation of LuxO. The *qrr* sRNAs are no longer expressed, and HapR is produced (66). In *V. cholerae*, QS controls many developmental phenotypes such as biofilms, virulence factor expression, extracellular protease production, and competence (21, 35, 45, 69). Biofilms and virulence are induced at low cell densities, whereas protease production and competence

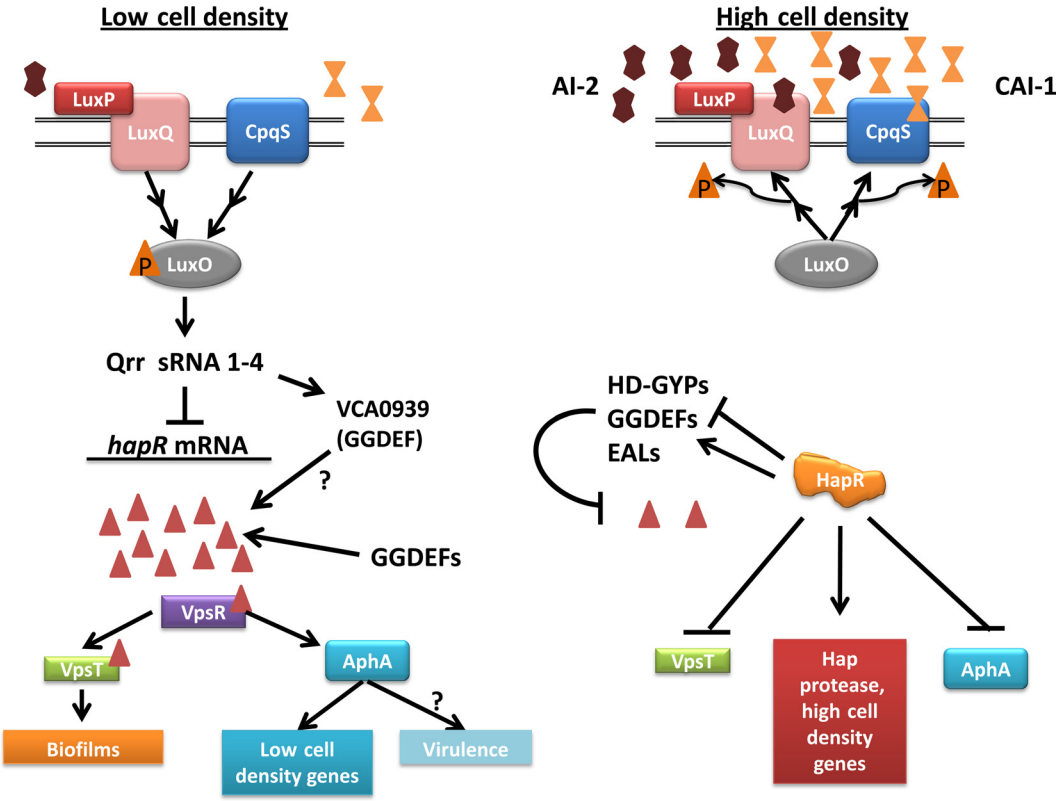

FIG 3 Control of c-di-GMP by the QS system of *Vibrio cholerae*. QS-mediated control of c-di-GMP in *V. cholerae* occurs at multiple levels. At low cell density, the levels of AIs AI-2 (brown double pentagons) and CAI-1 (orange double triangles) are low, causing the LuxPQ (pink) and CpqS (blue) histidine kinase receptors to ultimately phosphorylate the response regulator LuxO (gray; not all steps in this pathway are shown). Phosphorylated LuxO activates the expression of *qrr* sRNAs, which repress HapR expression by destabilization of *hapR* mRNA. HapR (orange) is the master high-cell-density regulator of *V. cholerae*. Qrr sRNAs also activate expression of VCA0939, a GGDEF domain-containing protein, by stabilizing its transcript. In the low-cell-density state, the levels of c-di-GMP (red triangles) in the cell are high. VpsR (purple) and VpsT (green), two transcriptional activators that directly bind to c-di-GMP, positively regulate biofilm genes. Also, expression of AphA (blue), the master QS low-cell-density regulator, is induced by VpsR and c-di-GMP to activate low-cell-density-expressed genes. Virulence factor expression is also induced by AphA but thought to be repressed by c-di-GMP, and this contradiction is not currently understood. At high cell densities, the increase in AI-2 and CAI-1 levels reverses the flow of phosphate in the QS cascade, leading to decreased *qrr* sRNA expression. The lack of *qrr* sRNAs increases HapR protein, which then regulates the transcription of multiple GGDEF, EAL, and HD-GYP enzymes, both positively and negatively, to decrease c-di-GMP levels in the cell. HapR also directly represses *vpsT* and *aphA* expression, decreasing biofilm formation, virulence factor expression, and low-cell-density gene expression. The expression of high-cell-density genes is increased by the presence of HapR.

induction occur at high cell densities (48). Repression of biofilm formation in the high-cell-density state is thought to be a mechanism of dispersal from mature biofilms in *V. cholerae*.

C-di-GMP signaling in *V. cholerae* has also been intensively studied. *V. cholerae* contains 61 predicted c-di-GMP synthesis/degradation enzymes, although a subset of these might be enzymatically inactive (18). In *V. cholerae*, c-di-GMP positively regulates biofilm development (5, 63) and negatively controls motility and virulence (37, 60, 64). Biofilm expression is highly induced by c-di-GMP through induction of the *vps* (vibrio polysaccharide) genes, while c-di-GMP represses expression of the flagellar biosynthetic genes (5, 37, 42). Transcription of virulence factor genes is also repressed by c-di-GMP, although the molecular mechanism by which this occurs is unknown (10). These results have led to a model whereby c-di-GMP levels are high in *V. cholerae* during environmental persistence where the organism exists primarily as a biofilm and repressed upon infection of humans (60).

In *X. campestris*, direct sensing of an AI activated an HD-GYP enzyme to decrease c-di-GMP levels at high cell density. *V. cholerae* is similar in that c-di-GMP levels are higher in the low-cell-density state and reduced at high cell density, but unlike the simple, direct sensing observed in *X. campestris*, QS modulation of c-di-GMP occurs at multiple levels throughout the *V. cholerae* QS signal cascade (71) (Fig. 3). The *qrr* sRNAs were shown to directly stimulate translation of the mRNA encoding the GGDEF VCA0939 protein independently of master regulator HapR (22). The induction of VCA0939 by *qrr* RNAs is consistent with a high intracellular concentration of c-di-GMP in the low-cell-density state, although a deletion of VCA0939 did not significantly affect biofilm formation (22). However, this result is not surprising, as single mutations in complex c-di-GMP signaling networks often do not exhibit strong phenotypes and the activity of VCA0939 remains to be tested.

QS control of c-di-GMP levels in *V. cholerae* also occurs downstream of the *qrr* sRNAs. Expression of HapR at high cell density controls the transcription of 14 different GGDEFs and EALs, ultimately resulting in decreased c-di-GMP levels and lower *vps* expression (71). Likewise, a mutation in *hapR* that caused a smooth-to-rugose transition in colony morphology led to higher levels of c-di-GMP (4, 71). Similarly, a screen to identify QS-regulated

genes showed that HapR regulated the transcription of four HD-GYP domain-containing proteins that may degrade c-di-GMP (22a). Increased production of the VCA0681 HD-GYP protein reduced c-di-GMP levels, decreasing *vps* gene expression and biofilm formation (22a).

In addition to directly modulating c-di-GMP concentrations at multiple levels, QS and the c-di-GMP regulatory machinery converge to control the expression of two key transcription factors in *V. cholerae*: AphA and VpsT. We recently determined that c-di-GMP induces expression of *aphA* in *V. cholerae* (58). As mentioned above, *aphA* is the master low-cell-density transcriptional QS regulator (51). In this context, c-di-GMP induction of the transcription of *aphA* makes sense, as c-di-GMP levels are higher at low cell density (71). C-di-GMP induction of *aphA* depends on the transcription activator VpsR, which requires direct binding to c-di-GMP to promote gene expression (58). In addition, QS also regulates *aphA* expression directly through HapR repression of the *aphA* promoter at high cell densities (35). The binding sites for HapR and VpsR overlap in the *aphA* promoter, and their binding is mutually exclusive (41). Therefore, QS and c-di-GMP regulation are integrated to control *aphA* expression at the level of transcription. Interestingly, this control of gene expression is also seen in regulation of *vpsT* expression, which is similarly regulated by HapR and VpsR using overlapping DNA binding sites (58, 71). The integration of QS and c-di-GMP signaling pathways functions as a regulatory checkpoint to combine multiple environmental inputs into *vpsT* and *aphA* expression.

It is currently unclear how virulence gene expression occurs in the low-cell-density state when c-di-GMP levels are high, as c-di-GMP is presumed to repress virulence factor expression (60, 61). Moreover, AphA, which is induced by c-di-GMP, positively activates virulence factor expression (56). These apparently contradictory statements highlight our lack of understanding regarding the regulation of virulence by QS and c-di-GMP. We speculate that specific *in vivo* host cues during infection might decrease c-di-GMP in the low-cell-density state, leading to increased virulence factor expression. As induction of AphA expression by c-di-GMP did not significantly impact virulence factor expression under laboratory conditions (58), this regulatory circuit might be more relevant to control of other low-cell-density QS targets.

The QS system of *V. cholerae* appears to modulate the c-di-GMP signaling pathway through alteration of c-di-GMP synthesis/degradation enzyme expression. Unlike findings with respect to *X. campestris*, no direct modulation of c-di-GMP synthesis/degradation enzyme activity by a *V. cholerae* AI has been described. It is important to remember that the overall activity of these enzymes depends on expression levels as well as on the proper environmental context. Thus, the QS system changes the distribution of c-di-GMP synthesis/degradation enzymes in the cell, but the ability of these enzymes to modulate c-di-GMP pools also depends on environmental signals. Why these different distributions of GGDEF, EAL, and HD-GYP enzymes are correlated with cell density and how these distributions increase the fitness of *V. cholerae* in low- versus high-cell-density environments are important issues for future research efforts.

TWO DISTINCT QS PATHWAYS IN *VIBRIO PARAHAEMOLYTICUS* MODULATE C-DI-GMP

V. parahaemolyticus, a close relative of *V. cholerae* that can also cause gastroenteritis, encodes two distinct QS systems, both of which influence the levels of c-di-GMP. The best-studied QS network in *V. parahaemolyticus* resembles that of *V. cholerae*, and this system controls c-di-GMP levels indirectly through alteration of c-di-GMP synthesis/degradation enzyme expression. Interestingly, many strains of this bacterium contain mutations that silence the canonical QS circuit (44). This QS system controls a number of behaviors in *V. parahaemolyticus*, including type III secretion, surface sensing, swarming, and a type IV secretion system (19). *V. parahaemolyticus* encodes homologs of all of the components of the *V. cholerae* QS regulatory pathway (28, 43). OpaR, the homolog of HapR, increases c-di-GMP levels at high cell density by affecting expression of 18 different GGDEF-, EAL-, and HD-GYP-encoding genes (19). It is possible that the OpaR-mediated increase of c-di-GMP is responsible for the higher expression of the capsular polysaccharide (*cps*) genes, higher DNA competency, and decreased cytotoxicity to human cells (19). Interestingly, this regulation is opposite that seen in *V. cholerae*, whereby HapR reduces c-di-GMP levels. Note that the levels of c-di-GMP were measured from bacteria either extracted from a broth culture for *V. cholerae* or grown on the surface of an agar plate for *V. parahaemolyticus* (19, 71), which could explain the observed difference.

Recently, the Scr system of *V. parahaemolyticus*, which has been studied for some time, was shown to be a novel QS system that modulates c-di-GMP levels (65). The Scr system closely resembles the Rpf system of *X. campestris* in that an AI molecule directly controls the activity of a c-di-GMP degradation enzyme. This QS system primarily controls surface growth phenotypes such as swarming and biofilm development. Swarming is an adaptation of many bacteria that allows movement in a lawn-like manner over a surface. In *V. parahaemolyticus*, swarming is facilitated by peritrichous flagella synthesized by the *laf* gene cluster (15).

Control of *laf* gene expression is mediated by the *scrABC* operon containing *scrA*, a gene encoding a pyridoxal phosphate-dependent enzyme that is implicated in AI biosynthesis, *scrB*, a periplasmic solute binding protein, and *scrC*, a GGDEF-EAL domain containing membrane-bound enzyme (Fig. 4) (6). The deletion of these genes leads to induction of biofilm formation and a loss of swarming phenotype, presumably due to a striking decrease in *laf* expression. Therefore, ScrABC positively regulates swarming and negatively regulates the *cps* operon that is responsible for extracellular polysaccharide production (6). ScrC functions as a DGC in the absence of ScrA and ScrB but acts as a PDE in the presence of ScrA and ScrB (16). High c-di-GMP levels activate *cps* expression and biofilm formation, whereas low c-di-GMP levels induce lateral flagellum biosynthesis and swarming (16).

Although it was clear that ScrC impacted biofilm formation and lateral flagellar production via modulation of c-di-GMP levels, the mechanism by which ScrA and ScrB modulated ScrC activity was a mystery until it was recently demonstrated that ScrA synthesizes an AI termed the S-signal. The S-signal is sensed by ScrB, a periplasmic solute binding protein which then modulates ScrC activity (65). At low cell densities, S-signal levels are low and ScrC presumably acts as a DGC to increase c-di-GMP levels, which induces *cps* gene expression. As the population density increases, S-signal accumulates and binds to ScrB, altering its interaction with ScrC. The S-signal alteration of this protein complex converts ScrC to a PDE, leading to a decrease in c-di-GMP levels and induction of *laf* gene expression and swarming (65). Thus, if

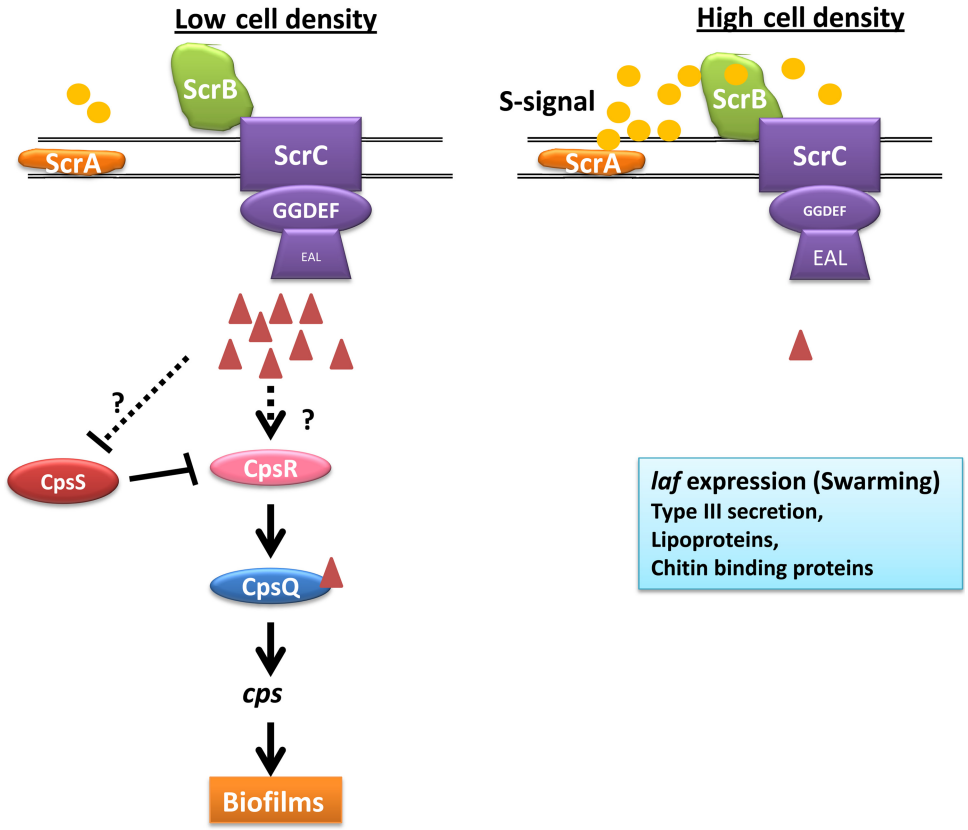

FIG 4 The ScrABC system of *Vibrio parahaemolyticus.* The Scr QS system of *V. parahaemolyticus* utilizes c-di-GMP to transmit the cell density information to the control of downstream gene expression. In this system, at low cell densities, the levels of S-signal (depicted by yellow circles) synthesized by ScrA (orange) are low. ScrB (green), the receptor for the S-signal, is in its unbound state, and ScrC (purple), the membrane-bound GGDEF and EAL domain protein, acts as a DGC, increasing c-di-GMP levels in the cell (red triangles). High c-di-GMP levels are sensed by CpsQ (blue), a c-di-GMP binding transcription factor, which activates downstream *cps* gene expression and induces biofilm development. CpsR (pink) is a transcription factor that induces expression of CpsQ, while CpsS (red) represses CpsR. The predicted connections between c-di-GMP, CpsS, and CpsR are depicted by dashed lines. At high cell densities, S-signal increases and ScrB binds the S-signal, changing its interaction with ScrC to convert it to a PDE to reduce c-di-GMP levels. This reduction in c-di-GMP levels leads to an increase in *laf* gene and lateral flagellar expression, type III secretion, and lipoprotein and chitin binding protein levels.

V. parahaemolyticus is present on a surface at a high cell density, swarming functions are induced by decreasing c-di-GMP levels. As swarming is a coordinated motility process, this QS system could ensure that sufficient *V. parahaemolyticus* bacteria are nearby to warrant coordinated motility. In addition, this density-dependent regulation may serve as a mechanism of surface colonization whereby bacteria at high cell density are stimulated to colonize new locations.

Microarray analyses revealed that ScrABC controls a regulon of more than 100 genes (17). This analysis also identified regulatory transcription factors as a part of the ScrABC regulon, one of which, CpsQ, is homologous to members of the VpsT family of transcription factors (Fig. 4) (17). Since VpsT is a c-di-GMP binding transcription factor in *V. cholerae*, it was hypothesized and subsequently shown that CpsQ binds to c-di-GMP and directly activates *cps* expression (17). Two additional regulators, CpsS and CpsR, were demonstrated to be involved in CpsQ-mediated control of *cps* (17, 20). CpsS is another VpsT homologue that functions to repress CpsR expression. CpsR, on the other hand, is a VpsR homologue that activates CpsQ expression. This regulation is reminiscent of control of biofilm development in *V. cholerae*, where two c-di-GMP binding proteins, VpsR and VpsT, are required for *vps* expression (Fig. 3). In *V. cholerae*, VpsR binds c-di-GMP to activate VpsT expression. VpsT then binds c-di-GMP to activate downstream *vps* gene expression, inducing biofilms (37, 58). CpsR is required for the induction of *cpsQ* expression in a *scrABC* mutant background, which is characterized by high c-di-GMP levels (17). CpsS and CpsR binding to c-di-GMP has not yet been examined, but it is likely that these transcription factors directly sense c-di-GMP. Many aspects of this pathway still need to be elucidated. It is unclear how *laf* gene expression is induced at low c-di-GMP levels or if CpsQ has a role in this process. Also, the chemical nature of the S-signal and the molecular mechanism by which ScrB and the S-signal control ScrC activity have not been determined.

Like the *X. campestris* and *V. cholerae* pathways, the Scr QS system of *V. parahaemolyticus* leads to high c-di-GMP concentrations at low cell density and low c-di-GMP concentrations at high cell density. If one considers biofilm formation to be a coordinated group behavior, this modulation of c-di-GMP by QS in these three bacteria is surprising, as it would be expected that high c-di-GMP levels, which promote biofilm formation, would be present at high cell density. For these regulatory pathways, the sensing of QS AIs by c-di-GMP signaling pathways might serve more as a dispersal

mechanism than as a mechanism for coordination of group behavior. However, the second QS system of *V. parahaemolyticus* is different in that c-di-GMP levels are increased at high cell density. It is not understood why two such divergent QS pathways, both of which alter the levels of c-di-GMP, exist in *V. parahaemolyticus*. These pathways appear to function antagonistically in linking cell density to c-di-GMP. It is possible that *V. parahaemolyticus* resorts to one or the other pathway depending on its environment, such as liquid versus surface growth. In some environments, QS is utilized as a signal to induce group biofilm formation, whereas in other environments, QS promotes surface colonization through induction of swarming.

IN THE BEGINNING: THE FIRST CONNECTIONS BETWEEN QS AND C-DI-GMP IN OTHER BACTERIA

The connections between QS and c-di-GMP in other bacteria have also begun to be elucidated, although these systems are not as well studied as the three systems described above. In *P. aeruginosa* strain PA14, the tyrosine phosphatase TpbA was shown to inhibit the activity of the TpbB GGDEF enzyme by dephosphorylation to reduce biofilm formation (68). TpbA is positively regulated by the Las QS system. Therefore, these studies suggest that QS negatively influences c-di-GMP production in *P. aeruginosa*, but the concentration of c-di-GMP at different QS states has not yet been determined. The connections between QS and c-di-GMP were recently also examined in *Aeromonas hydrophila* (36). The QS system of *A. hydrophila* is not well characterized, but it resembles the QS systems of both pseudomonads and *Vibrio* species (36). Although c-di-GMP was not directly measured at different QS states, a connection between these two systems was postulated based on the observation that induction of biofilm formation by overexpression of a DGC was dependent on an intact AI-2 QS system (36). This result suggests that these two systems might be connected in *A. hydrophila*; however, it remains to be shown whether QS and c-di-GMP are combined to control downstream phenotypes or function as parallel signaling pathways in this bacterium.

CONCLUDING REMARKS

The connections between QS and c-di-GMP can be considered to be either direct, as in the case of *X. campestris* and the Scr system of *V. parahaemolyticus*, whereby a AI directly controls the activity of a c-di-GMP synthesis or degradation enzyme, or indirect, as in the case of *V. cholerae* and the homologous *V. parahaemolyticus* QS system, whereby the expression of c-di-GMP synthesis/degradation enzymes is controlled by QS. In direct control, modulation of c-di-GMP levels by QS is less dependent on environmental conditions, as the AI itself is the environmental cue. Alternatively, indirect control would be more sensitive to the local environment, as the activity of the target DGCs and PDEs would still be dependent upon the presence of their cognate environmental cues. These distinct architectures allow the evolution of regulatory pathways with various degrees of integration with other environmental factors.

How widespread are the connections between QS and c-di-GMP signaling? Are connections between QS systems and c-di-GMP exceptions or the norm? Thus far, the integration of QS and c-di-GMP has been demonstrated only in the few organisms described above, but we predict that this list will continue to grow. In our opinion, the major function of c-di-GMP is to sense, integrate, and transduce environmental cues to properly regulate the transition from sessility to motility, as well as other behaviors just beginning to be characterized. One can view AIs and the QS systems that sense them as mechanisms that relay one component of the environment: local cell density. Therefore, it is not surprising that this information would be integrated into the modulation of c-di-GMP pools in the cell as described in the examples above.

In all of these systems, c-di-GMP is epistatic to QS inputs. In other words, these regulatory arrangements highlight the ability of c-di-GMP levels to override QS control based on additional environmental signals. For example, in *V. cholerae*, overproduction of an active GGDEF enzyme alleviates repression of biofilm gene expression in the high-cell-density QS state (71). Other systems seem to parallel this arrangement. If one considers that c-di-GMP signaling systems are integrating multiple pieces of information regarding the local environment, this view makes logical sense. This regulatory architecture allows specific environments that strongly promote biofilm formation or other c-di-GMP-controlled behaviors to override QS regulation of these processes. Therefore, we propose that QS signals appear to be one environmental component, of many, integrated into the broader cellular c-di-GMP signaling system (Fig. 1). QS is a fundamental mechanism of environmental sensing in bacteria, and we predict that similar regulatory connections whereby c-di-GMP signaling systems respond to AIs either directly or indirectly are present in other bacterial species and await discovery.

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Chris Waters received a B.S. in Genetics from The University of Kansas in 1997, researching pathogenic *Escherichia coli* with Eric Elsinghorst. He obtained his Ph.D. from the University of Minnesota in 2002, studying the *Enterococcus faecalis* pheromone-inducible plasmids with Gary Dunny. His postdoctoral training with Bonnie Bassler at Princeton University sought to understand chemical signaling in *Vibrio harveyi* and, later, *Vibrio cholerae.* Chris has been an assistant professor in the Department of Microbiology and Molecular Genetics at Michigan State University since 2008. He and his laboratory colleagues seek to elucidate the mysteries of quorum sensing and cyclic di-GMP signaling in *V. cholerae* when not undertaking bowling challenges, exploring on canoe trips, or producing zombie movies.

Disha Srivastava is a doctoral candidate working with Chris Waters at Michigan State University. She received her B.S. in Biomedical Science and M.S. in Plant Molecular Biology at the University of Delhi, Delhi, India. Disha joined the Biochemistry and Molecular Biology program at Michigan State University in 2008. Her research in *Vibrio cholerae* seeks to understand the molecular mechanisms by which cyclic di-GMP regulates biofilm formation and motility and the interconnections between cyclic di-GMP signaling and quorum sensing.